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Award Number: DAMD17-01-1-0581

TITLE: Role of the Epidermal Growth Factor Receptor Variant (EGFRvIII) in Radiation Response of Human Breast Cancer Cells

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REPORT DATE: August 2002

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 2002	Final (1 Aug 01 - 31 Jul 02)

4. TITLE AND SUBTITLE
Role of the Epidermal Growth Factor Receptor Variant (EGFRvIII) in Radiation Response of Human Breast Cancer Cells

5. FUNDING NUMBERS
DAMD17-01-1-0581

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
report contains color

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13. ABSTRACT *(Maximum 200 Words)*

A variant of EGFR, EGFRvIII, consistitutively activates signal transduction pathways associated with radioresistance. In order to determine whether expression of EGFRvIII confers radioresistance in human breast cancer or breast epithelial cell lines, we expressed EGFRvIII in a normal mammary epithelial cell line (MCF10A) and in a breast cancer cell line (SUM44). Expression of EGFRvIII caused EGFRvIII caused constitutive tyrosine phosphorylation of itself and HER2, accompanied by constitutive activation of p44/42 MAPK and Akt. EGFRvIII expression did not alter survival after single doses of radiation ranging from 1-10Gy. However, EGFRvIII conferred resistance to fractionated radiotherapy (3 fractions of 2Gy). This protective effect was inhibited by pre-treatment of the cells with an EGFR/HER2 kinase inhibitor that inhibited both the constitutive activation of EGFRvIII and HER2 and the constitutive activation of the p44/42 MAPK downstream signal transduction pathway, but not the PI3-K/Akt pathway. Thus, EGFRvIII expression induces resistance to fractionated radiotherapy, and the resistance correlates with p44/42 MAPK signaling in this breast epithelial/cancer model. Further studies are warranted to investigate the nature of the resistance to fractionated radiotherapy (clinically relevant delivery of radiotherapy) and to investigate the role of dual EGFR/HER2 inhibitors as radiosensitizers.

14. SUBJECT TERMS
radiation sciences, epidermal growth factor receptor, breast cancer

15. NUMBER OF PAGES
11

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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INTRODUCTION:

There is increasing evidence that the epidermal growth factor receptor (EGFR) family plays an important role in radioresistance of human breast cancer. Over-expression of HER2 is associated with local recurrence of breast cancer after radiotherapy [1]. EGFR activation is associated with increased radioresistance *in vitro* [2,3], and EGFR over-expression is associated with radioresistance in clinical tumor specimens [4,5]. Kinase inhibitors or antibodies that perturb EGFR or HER2 signaling show promise as radiosensitizing agents both in pre-clinical and clinical studies (reviewed in [6]. In gliomas, rearrangements of the EGFR gene were identified, including a commonly occurring rearrangement that results in loss of exons 2-7 of the extracellular domain [7]. This variant, EGFRvIII, constitutively activates signal transduction pathways associated with proliferation and survival, and may perturb the normal interactions of the endogenous EGFR family members [8,9]. EGFRvIII has been reported to be expressed in a large percentage of human breast cancers and to play an important prognostic role [10]. Expression of EGFRvIII confers resistance to chemotherapeutic agents in gliomas via modulation of the apoptotic pathway, and the downstream signal transduction pathways constitutively activated in some EGFRvIII-expressing cells, PI-3-kinase and MAPK, have been implicated in radioresistance [5,11]. Thus, there is a distinct possibility that EGFRvIII may mediate radioresistance or affect activation of other EGFR family members thought to be involved in radioresistance. If so, there is potential for improving radiation response by using EGFRvIII inhibitors, which are currently in advanced stages of development for clinical use, as radiosensizing agents that would have a high specificity for tumor as opposed to normal tissues.

BODY:

1) Creation and Characterization of EGFRvIII-expressing Cell Lines

The EGFR has been implicated in radiation response modulation when activated by ligand and when activated by ionizing radiation. Unlike HER2, which is constitutively active when over-expressed, over-expression of EGFR has not been shown to render it constitutively active in the absence of ligand. However, a mutant EGFR, present in gliomas and reportedly in a large percent of breast cancer, is constitutively active in the absence of ligand. This EGFR variant, designated EGFRvIII, resembles the oncogenic v-erbB, in that it has deleted a significant portion of the extracellular, ligand binding domain.

In order to determine whether expression of EGFRvIII confers radioresistance in human breast cancer cell lines or normal breast epithelial cell lines, we expressed EGFRvIII in a normal mammary epithelial cell line that expresses normal levels of

EGFR, HER2, and HER3 (MCF10A) and in a breast cancer cell line that expresses all EGFR family member4s except EGFR (SUM44). Both cell lines grow in serum-free, defined growth factor condition, and EGFRvIII did not affect the proliferative rate in the presence of necessary growth factors. However, in growth factor depleted medium, EGFRvIII expression conferred EGF and IGF independence in MCF10A cells. Expression of EGFRvIII enabled soft agar growth of MCF10A cells (SUM44 cells grow in soft agar regardless of EGFRvIII expression).

Expression of EGFRvIII caused constitutive tyrosine phosphorylation of EGFR in MCF10A cells and of HER2 in MCF10A and SUM44 cells (Figure 1A). Expression of EGFRvIII also induced constitutive activation of p44/42 MAPK and Akt, but did not alter activation of p38 MAPK. EGF-induced activation of SAPK/JNK was reduced in EGFRvIII-expressing cells (Figure 1B).

Figure 1A. Constitutive activation of EGFRvIII and HER2.

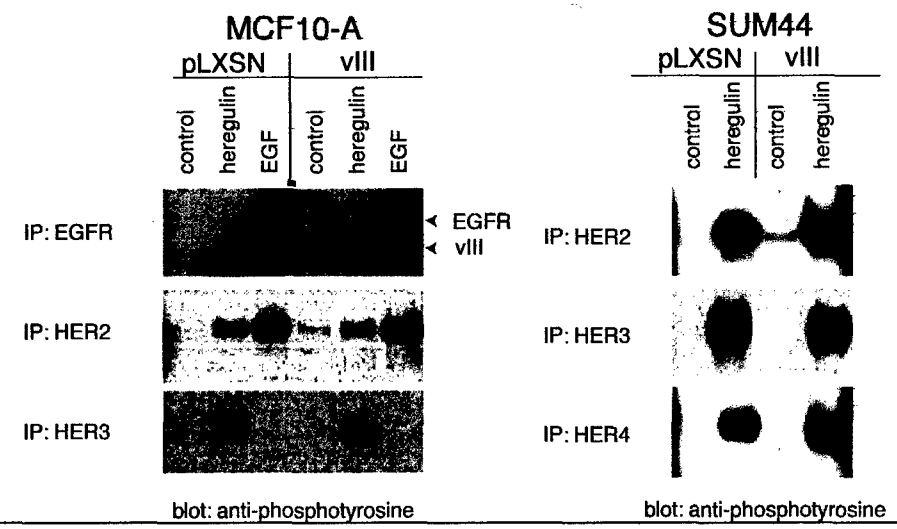
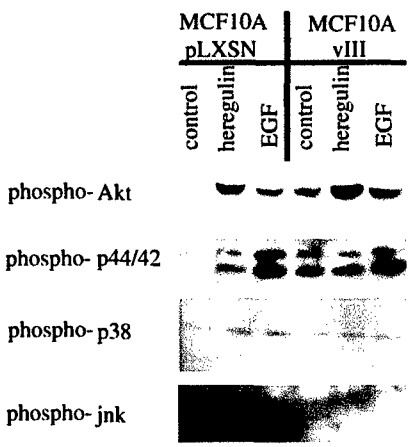
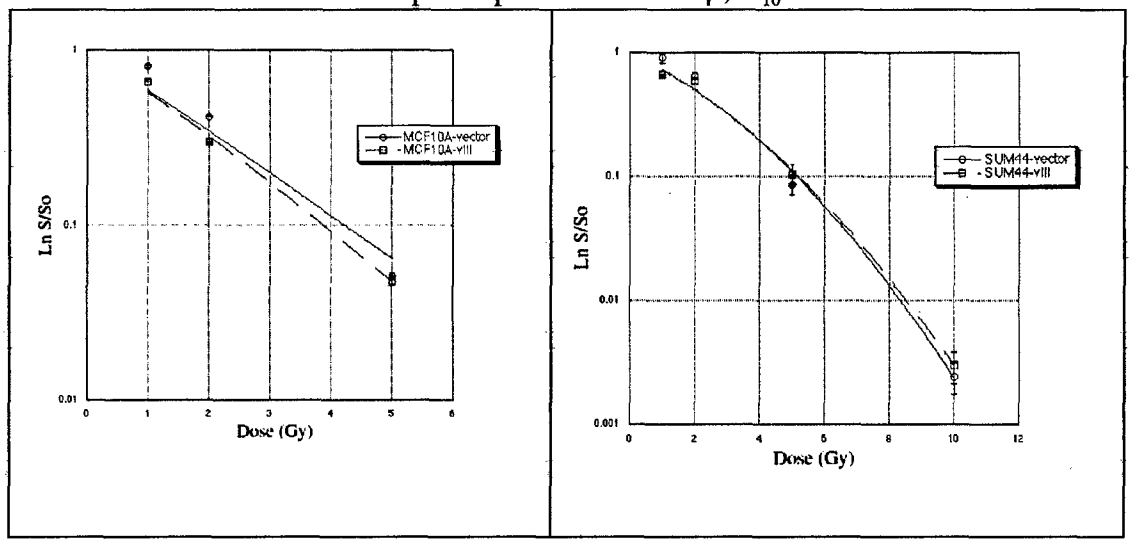


Figure 1B. Constitutive activation of Akt and p44/42 MAPK.



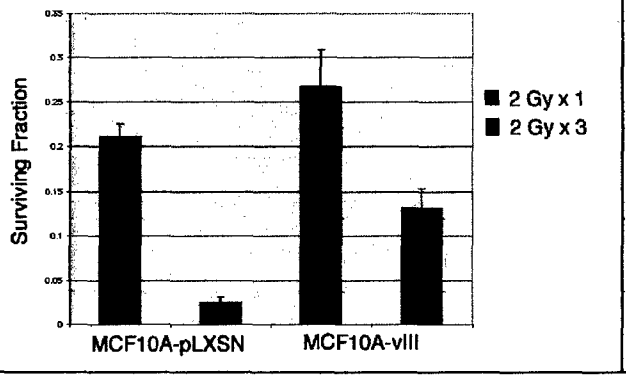
2. EGFRvIII effect on radiation response

To determine the effect if any, of the EGFRvIII-induced constitutive signaling on radiation response, standard colony forming assays were performed with either vector-control SUM44 cells (SUM44-pLXSN) vs. SUM44 cells expressing EGFRvIII (EGFRvIII) and vector-control MCF10A cells (MCF10A-pLXSN) or MCF10A cells expressing EGFRvIII (MCF10A-vIII). Figure 2 demonstrates that there was no difference between vector control and EGFRvIII-expressing cells in response to single dose ionizing radiation over a range of doses for either cell line, nor were there differences in the radiation response parameters of α/β, D_{10} or Do.



Clinically, radiation therapy is delivered using multiple fractions of lower dose to enable normal cells to arrest and repair between fractions. EGFR family member signaling has been implicated in repair and arrest functions, so we wished to determine whether EGFRvIII expression affected response to fractionated radiotherapy. Vector control or EGFRvIII-expressing cells were treated with either a single dose of 2 Gy or 3 fractions of 2Gy each. There was no difference in colony formation after a single dose of 2 Gy, as expected from the above studies, but EGFRvIII-expressing cells demonstrated radioresistance compared with vector control cells when treated with 3 fractions of 2 Gy (Figure 3).

Figure 3. Resistance of fractionated radiation.



3. Effect of inhibition of constitutive activation of EGFRvIII and HER2 in EGFRvIII-expressing cells by a dual EGFR/HER2 kinase inhibitor, GSK702016.

To determine whether constitutive activation of the EGFRvIII and/or HER2 was responsible for the apparent radioresistance to fractionated treatment in the EGFRvIII-expressing cells, a small molecule tyrosine kinase inhibitor that inhibits both EGFR and HER2, GW702016, was employed (kindly supplied by Glaxo, Inc.). GW702016 inhibited both EGFRvIII and HER2 constitutive activation by 1uM (Figure 4A), and also inhibited the subsequent constitutive activation of p44/42 MAPK, but did not inhibit constitutive activation of Akt (Figure 4B).

Figure 4A. Inhibition of EGFRvIII and HER2 activation by GW702016.

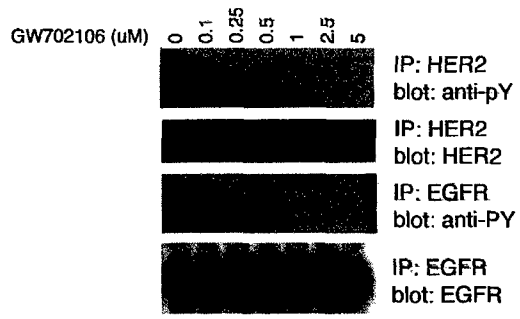
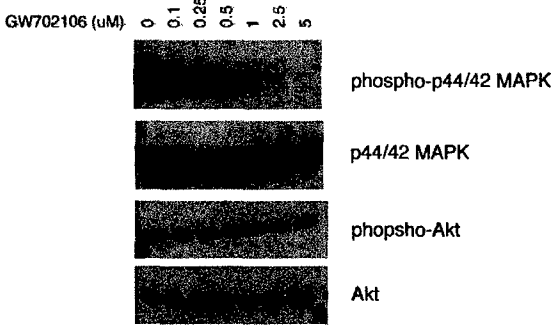
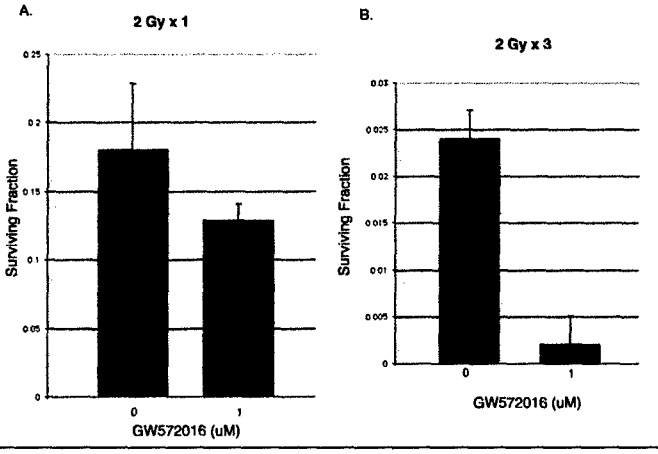


Figure 4B. Inhibition of downstream stp's by GW702016.



To determine whether GW702016 inhibition of constitutive EGFRvIII and HER2 and subsequent inhibition of p44/42 MAPK activation in EGFRvIII-expressing cells, cells were treated with 1uM GW702016 30 minutes prior to a single dose of 2Gy or 3 doses of 2Gy radiation. GW702016 did not significantly affect response to single dose radiation, but markedly sensitized cells to fractionated radiation (Figure 5).

Figure 5. Effect of GW702016 on radiation response to single dose or fractionated radiation.



While our preliminary data in the MCF10A-EGFRvIII expressing line indicates that GW702016 reverses the EGFRvIII-mediated resistance to fractionated radiotherapy, these experiments need to be confirmed with a cell line that has more consistent results on clonogenic survival assays. To this end, we are in the process of confirming the fractionated radiation response in SUM102-expressing EGFRvIII cells.

KEY RESEARCH ACCOMPLISHMENTS:

- Creation of EGFRvIII-expressing breast epithelial and breast cancer cell lines.
- Demonstration that EGFRvIII expression induces constitutive EGFRvIII and HER2 activation
- Demonstration that EGFRvIII expression results in constitutive p44/42 MAPK and Akt activation.
- Demonstration that EGFRvIII expression does not affect radiation response to single dose radiation, but does induce radioresistance to fractionated radiotherapy at clinical relevant fraction doses.
- Demonstration that GW702016 inhibits constitutive activation of EGFRvIII and HER2.
- Demonstration that GW702016 inhibits constitutive activation of p44/42 MAPK but not Akt.
- Demonstration that GW702016 reverses EGFRvIII-mediated radioresistance to fractionated radiation.

REPORTABLE OUTCOMES:

- Manuscript "Epidermal growth factor variant (EGFRvIII) expression in a normal human breast epithelial cell line induces growth factor independence, constitutive activation of downstream signal transduction pathways, and anchorage-independent growth" in preparation, plan submission to Oncogene.
- If the radiation response results are confirmed in SUM102-EGFRvIII cells, we will prepare a manuscript reporting the resistance to fractionated radiotherapy, reversal of resistance by GW702016, and correlation of reversal of radioresistance with inhibition of p44/42 MAPK, but not Akt.

CONCLUSIONS:

Our results indicate that the EGFRvIII induces resistance to fractionated radiotherapy at clinically relevant dose per fraction, and that resistance correlates with constitutively active EGFRvIII and HER2 signaling through p44/42 MAPK. Furthermore, inhibition with GW702016, a dual EGFR/HER2 tyrosine kinase inhibitor reverses the radioresistance.

"So what?" While EGFR and HER2 activation have been implicated in radioresistance, the role of EGFRvIII is unknown. Our findings indicate that EGFRvIII induces constitutive activation of an EGFR-like signal and a HER2 signal. If the resistance to fractionated radiotherapy is confirmed in the SUM102-EGFRvIII cell line, our results will provide novel evidence for a role of EGFR family signaling in resistance to fractionated radiotherapy, providing a nidus for investigation of the mechanism(s) involved in resistance to therapeutically relevant fractionated radiotherapy. Furthermore, our findings demonstrate that a new, reversible, dual EGFR/HER2 kinase inhibitor inhibits the constitutive activation of EGFRvIII, HER2, and downstream p44/42 MAPK. Our results provide rationale for further investigation of small molecule tyrosine kinase inhibitors as radiosensitizers, particularly in EGFRvIII-expressing tumors, but also in tumors with constitutive EGFR or HER2 signaling via other mechanisms such as over-expression or autocrine activation. Finally, our results raise the intriguing possibility that inhibition of constitutive p44/42 MAPK may correlate with radiosensitization of EGFRvIII-expressing tumors, which, if confirmed in correlative trials may establish p44/42 MAPK as a predictor of response.

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APPENDICES:

None.

BIBLIOGRAPHY:

Manuscript "Epidermal growth factor variant (EGFRvIII) expression in a normal human breast epithelial cell line induces growth factor independence, constitutive activation of downstream signal transduction pathways, and anchorage-independent growth" in preparation, plan submission to Oncogene.

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